PARALLEL MECHANISMS IN THE PATHOGENESIS OF ATHEROSCLEROSIS AND AMYLOIDOSIS

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INTRODUCTION

Lesions of amyloidosis were found in this laboratory in the spleen and kidneys of a rabbit on a high cholesterol diet for 12½ months. [1]

This disease has previously been known to occur following prolonged cholesterol feeding. Hoffman et al [2] reported the development of amyloidosis or paramyloidosis in 7 out of 18 rabbits (39%) fed 3 - 12 gm. of cholesterol each week. Deposits occurred as early as 12 weeks while in some rabbits none occurred after two years of cholesterol feeding. The amorphous eosinophilic deposits more often than not stained negatively for amyloid using crystal violet and Congo red stains. Significant amounts of lipids within glomeruli were brought out by Sudan IV staining of frozen sections in two out of seven positive rabbits. Hyperglobulinemia of the beta fraction and hypercholesterolemia were both present. [3]

Johansson [4] considers the problem of the relationship of amyloidosis and arteriosclerosis and the influence of deranged lipid and protein metabolism which appears in both diseases. He refers to a personal communication of Hass [5] who produced nephrosis-like lesions in rabbit kidneys while the animals were on a high cholesterol diet.

The sites of deposition of amyloid include the subintimal regions of small arteries. [6] These sites, of course, are identical with the presumed sites of deposition of beta-lipoproteins in arteriosclerosis. This morphological similarity suggests that the artery wall may respond in an identical way to widely disparate macromolecular compounds, namely, glycoproteins in amyloidosis and lipoproteins in arteriosclerosis.

These two disease processes have been studied in numerous laboratory animals. Amyloid deposits have been produced in rabbits, [7] horses, [8] hamsters, [9] guinea pigs, [10] mice, [11] and cattle. [12] Various materials have been used to induce the formation of amyloid, namely, non-infectious antigens such as tetanus toxoid, streptococcus toxins, diptheria toxin, and tuberculin. [13] Heat-killed pneumococci were found ineffective. Protein compounds such as sodium caseinate have provoked amyloid to form. [14, 15] Vitamin C deficiency diets have also resulted in experimental amyloidosis. [10] Of the infectious agents, the tubercle bacillus has been most frequently used as an etiological agent. [13] Amyloid has even been reported to arise spontaneously in normal dogs [12] and in occasional strains of aged mice. [16] Nevertheless, the effectiveness of these numerous agents varies from one investigator to another. For example, Grayzel was able to induce amyloid formation in mice with nutrose, [15] whereas Hass was unable to do so when he injected the material less frequently but over a greater period of time. [13]

One of the most usual ways of producing arteriosclerosis is by feeding high cholesterol diets to various experimental animals. This type of diet produces the most notable changes in the rabbit, whereas the changes are less obvious in rats and guinea pigs and are difficult to produce in carnivores such as dogs and omnivores. The early lesions of atherosclerosis in man are in most respects analogous to the lesions found in rabbits.

Thus, we can see that the rabbit is a suitable experimental animal for producing both the lesions of amyloidosis and those of arteriosclerosis, each by an "independent" method. According to Calkins, the lesions of amyloidosis can readily be produced in rabbits following at least two months

of subcutaneous injections of sodium caseinate. [17] Arteriosclerosis can readily be produced by the addition of egg yolk to the diet as is usually done in this laboratory. Here we should have a ready model to study the morphological interrelationships of amyloidosis and arteriosclerosis with these two diseases developing simultaneously in the same experimental animal.

In the experiments to be reported, rabbits received long-term injections of sodium caseinate to produce amyloidosis at the same time that they were receiving a cholesterol-rich diet to produce experimental atherosclerosis. It was the plan of the experiment to study these animals morphologically to ascertain whether or not in these conditions excessive amounts of lipid would accumulate at sites of amyloid deposits, thus relating the pathogenesis of amyloidosis and of atherosclerosis.

METHODS & MATERIALS

In the first experiment (Series I), 18 albino rabbits received subcutaneous injections of sodium caseinate solution twice a week. The albino rabbits, also known as New Zealand whites, weighed approximately 3 kilograms. A 10 percent solution of sodium caseinate (from Nutritional Biochemical Corporation, Cleveland, Ohio) was prepared using sterile precautions. A fresh solution was mixed each week in a Waring blender with normal saline as the solvent. It was kept in a refrigerator between injections. Five milliliters of this solution were injected subcutaneously into a different site on the back each time. The first 12 rabbits in this series (C-E 1 through 12) were fed two egg yolks every two days mixed with the usual pellet diet. The other 6 rabbits (C-C 1 through 6) received only pellets and water ad libitum in their diets.

The second experiment (Series II), involved 12 albino rabbits treated in a similar way except that a 15 percent sodium caseinate solution was employed in an effort to accelerate the process of amyloid deposition. On this occasion, the caseinate was obtained from Matheson, Coleman and Bell, Division of Matheson Company, East Rutherford, New Jersey. Rabbits W-G 1 through 6 were fed egg yolks and pellets while rabbits W-G 7 through 12 had no egg yolks added to their diet.

Cultures for aerobic and anaerobic bacteria were obtained on the exudate from the subcutaneous nodules that formed at injection sites and on the caseinate solution. The material was plated out on blood and desoxycholate agar. Urine analyses were performed on several rabbits.

Serum levels of lipids, proteins, and non-protein nitrogen were determined on all rabbits at the beginning of and during the course of the experiments. The following methods were employed: a modified Schoenheimer-

Sperry methods for free and total cholesterol, [18] a modified Youngburg procedure for lipid phosphorus, [19] and the method of Man and Gildea for fatty acids. [20] Serum protein and NPN levels were estimated from nitrogen determinations by the usual micro-kjeldahl procedure and steam distillation of the ammonia formed.

It was planned that animals would be sacrificed for histological study at monthly intervals. Several animals died during the initial phase of the experiment; consequently, few rabbits had to be sacrificed to obtain sequential material for study. The lack of histological evidence of amyloidosis in the first 5 months led to the prolongation of the experiment to nine months.

The rabbits were sacrificed by the intravenous injection of nembutal. At autopsy, the following organs were removed for histological study: heart, aorta, spleen, liver, kidneys, lungs, subcutaneous nodules, and in several rabbits, the eyes and adrenal glands. The tissues were fixed in 10 percent neutral formalin. Paraffin sections were stained routinely with hematoxylin and eosin. Special stains utilized included crystal violet, Congo red, periodic acid-Schiff, Masson trichome, and phosphotungstic acid hematoxylin stains. Frozen sections were made and stained with sudan IV routinely for lipids.

The rabbit diet consisted of pellets of Purina Rabbit chow checkers. Its composition was as follows: crude protein not less than 20.0 percent, crude fat not less than 2.0 percent and crude fiber not more than 16.0 percent. Vitamin and mineral salt supplements were also present. Those rabbits fed with egg yolk received approximately 40 gms. of egg yolk, that is, 8 gms. of cholesterol [21] every other day.

RESULTS

Two series of experimental animals were studied. In the first series, rabbits C-E 1 through 12 were fed egg yolk supplements and were injected subcutaneously with a 10 percent solution of sodium caseinate, while rabbits C-C 1 through 6 were only injected with sodium caseinate. From Table 1, it is evident that only 4 out of 18 rabbits developed amyloidosis. This occurred after a period of 9 months. Rabbits C-E 1 (Fig. 2) and C-C 4 had massive perifollicular deposits in the spleen and less impressive deposits in the renal glomeruli, in their basement membranes, and around the tubular basement membranes. Rabbits C-E 8 and 10 had small perifollicular deposits in the spleen accompanied by reticulum cell hyperplasia. Their kidneys were not microscopically involved.

These results are in marked contrast to those of Cohn, Calkins and Levene [17] who reported amyloidosis in 100 percent of rabbit spleens after 3-4 months and 100 percent of rabbit kidneys after 4-5 months. The methods in both cases were similar except that these workers obtained their caseinate from a different supplier and that their injected solution was more viscous. [22]

In an effort to reproduce Cohen *et al's* method more precisely, sodium caseinate was obtained from the Matheson Company. Furthermore, the concentration of caseinate solution was increased to 15 percent in order to make the solution more viscous, although Cohn *et al's* solution was sufficiently viscous at 10 percent. Higher concentrations than 15 percent were too viscous to inject.

A second series of rabbits was then started. Rabbits W-G 1 through 6 were fed egg yolk and were injected with a 15 percent solution two times a week, while rabbits W-G 7 through 12 were injected with sodium caseinate and

received no diet supplements. In this series, amyloid was produced in 5 out of 12 rabbits (see Table 2). This is a 42 percent yield as opposed to a 22 percent yield in the first series. Furthermore considerable deposits in the spleen occurred as early as 2½ to 4 months in rabbits W-G 4, 5 and 8. Out of the last 5 animals sacrificed at 5½ months, only two (W-G 10 and 11) had amyloid deposits evident. Rabbit W-G 10 had involvement of the kidney alone, while rabbit W-G 11 had amyloid in both the spleen and the kidney. The spleen was involved by itself in rabbits W-G 4, 5 and 8. Thus, a higher and earlier experimental yield was produced by increasing the concentration of the caseinate solution from 10 to 15 percent. Yet, the 100 percent yield of Cohen and his associates was still not obtained. Differences due to brand of sodium caseinate were not thought to be significant in increasing the incidence or severity of amyloidosis.

The liver, heart, aorta, lungs, and skin had no amyloid deposits in any of the rabbits studied. Several eyes and adrenals were examined; they contained no amyloid.

Special stains were done to establish the nature of the amorphous eosinophilic deposits considered to be amyloid in the hematoxylin and eosin stain. Crystal violet stains showed a fast-fading (15 minutes) violet-red metachromasia where massive deposits were present. The reaction was more marked in the positive spleens and kidneys from the second experimental series than those from the first. Congo red stains revealed these deposits to be more orange than the surrounding tissue but not bright orange or brick red. Here there was no difference in staining between the egg yolk fed rabbits and the non-supplemented animals. PTH stains were unrevealing. A few Masson

preparations indicated that the amorphous deposits were probably not fibrin. PAS stains were positive, but not diagnostic as any 1:2 glycol group will stain positively. Consequently, except for the crystal violet and the Congo red these special stains were not extremely helpful in defining the observed deposits as they are classically described.

The perifollicular deposits in the spleen and the amorphous deposits in the glomeruli of the kidney contained large amounts of lipid when stained with Sudan IV (Figs. 3 & 4). The spleens and glomeruli of amyloid-negative animals did not have these lipid deposits. PAS-positive granules were noted in lipophages. These were later established as normally present in lipophages.

In the hearts of egg yolk fed rabbits evidence of atherosclerosis, namely, foam cell infiltration of the intima and partially occluding fibrous plaques appeared in the majority by 3½ months. The aortas of similar animals showed fatty streaks when seen at 1-2 months. Later, other rabbit aortas exhibited raised plaques. These exhibited calcification in those rabbits sacrificed at the termination of the experiment. No amyloid deposits were identified in any of these lesions. In the blood of these rabbits as shown in Table 3, the total cholesterol ultimately rose to the markedly elevated range of 400-1, 150 mg. percent. The free cholesterol fraction was usually less than 50 percent of the total. The levels of fatty acids and of lipid phosphorus were usually at least two times the levels found in those on a cholesterol-free diet. Normal values for rabbits are found at the bottom of Table 4. [23]

The hearts and aortas of those rabbits without egg yolk diet supplements every other day were without the usual stigmata of atherosclerosis

except in a few instances. Rabbit C-C 5 died early in the experiment and was found to have a large calcified plaque at the base of the aorta. This probably developed spontaneously. Rabbit C-C 1 had one small fatty streak at a branch point in the aorta. Its total cholesterol was initially elevated in the experiment, but later had declined to normal.

Aside from these the cholesterol levels of non-egg yolk fed rabbits in the first series were normal. However, in the second series, the cholesterol levels were elevated in animals injected with caseinate but on low fat diets (see Table 4). Often, the free cholesterol fraction was one-tenth of the total rather than the usual one-quarter to one-half. Fatty change of the liver was not only seen in animals on an egg yolk supplemented diet, but also in two animals on a low fat diet (rabbits C-C 4 and W-G 7). Foam cells were seen at the periphery of the nodules in both types of experimental animals. This was more obvious in the second series. All of these findings raise the question of an associated disorder of lipid metabolism in amyloidosis.

Approximately, half of the animals died and half were sacrificed in each experimental series. Microabscesses were seen occasionally in the liver, spleen, kidneys, lungs or subcutaneous nodules in animals that died and in those sacrificed. Nodules developed on the backs of all rabbits at the different sites of injection by 3-4 months. These occurred despite swabbing the backs with 70 percent alcohol prior to the injection and despite sterilization of the needles and syringe. Cultures obtained on the thick, yellow purulent material aspirated from the nodules grew out bacteria in one-third of the cases cultured. The organisms seen were beta-hemolytic streptococcus (W-G 6), staphylococcus albus (C-E 10) and aureus (C-E 1 and W-G 9) or

Escherichia coli (W-G 11). Only one rabbit (W-G 6) that had an abscess in an organ had a positive nodule culture. Cultures were not obtained on rabbits C-E 9, C-C 2 and 5, and W-G 1. They all had microabscesses in at least one organ. The culture on rabbit W-G 12 was negative. There were other types of infections. The livers of many rabbits were the seat of a mild, chronic pericholangitis. This is commonly found in laboratory rabbits and is usually due to the fungus, Coccidioides. Pneumonia occurred rarely (rabbits W-G 1 and 6). Bacterial colonies were seen microscopically to line the wall of the abscesses on the backs of rabbits only once (rabbit W-G 1). Overall, however, there was no good correlation between the presence of infection and the incidence or severity of amyloidosis.

It was of interest that anaphylactic reactions apparently precipitated death in a few instances (rabbits C-E 2 and 6, and W-G 7); also that there was evidence of hypersensitivity in several animals (rabbits C-E 4, C-C 3 and 5, W-G 3, 6, 7, 9, 10, and 11). Following the usual injection of 5 mLs. of caseinate solution, the rabbits noted became hyperactive 5-10 minutes later, wheezed, convulsed, became prostrate and died. These episodes suggested a foreign protein reaction of an anaphylatic type. They did not occur in rabbits which survived beyond 2½ months from the start of the experiment. The lesions of hypersensitivity occurred in the heart (Fig. 1) in all instances but one (the lung of rabbit W-G 6). They consisted occasionally of fibrinoid necrosis or more often of perivascular infiltrates of the coronary arteries with polymorphonuclear leucocytes in the early stages or mononuclear cells later. The coronary arteries were frequently thickened and scarred in many of these rabbits. Myocarditis and focal fibrosis of the myocardium was infrequently

seen and showed no preference for the egg yolk fed or non-diet supplemented animals.

Thus, many different factors were responsible for the 50 percent mortality in these experiments. Infection, hypersensitivity reaction and atherosclerosis were probably the major causes of death. Amyloidosis was seldom the sole factor.

An isolated finding was that of a cholesteatoma - an accumulation of cholesterol ester crystals in a circumscribed mass of fatty and fibrous tissue - located in the liver of rabbit C-E 10.

The lungs in nearly every rabbit were congested and edematous in varying degrees. Other significant abnormalities of the lungs were noted earlier.

Amyloidosis is usually associated with a reversal of the albumin/globulin ratio and with renal damage. The serum determinations are found in Tables 3 and 4. In the two experimental series, many of the A/G ratios became inverted near the termination of the experiment. NPN levels never went above 50 mg. percent. Thus, none were markedly elevated. Furthermore, there was no correlation between the microscopic renal damage and NPN levels. However, a few random urine analyses done on the second series had some prognostic value when the findings were extreme. For example, rabbit W-G 11 had 3+ proteinuria and later was shown to have marked amyloid deposits in the kidney. Also, rabbit W-G 6 had 10-20 WBCs per HPF. It later had a positive nodule culture with beta-hemolytic streptococci, although this organism is not usually associated with pyelonephritis in man. The other urine specimens had the following characteristics: alkaline pH, 0-2+ proteinuria, 0 glycosuria, and considerable amounts of amorphous phosphates.

Sudan IV stains were done on all rabbits with amyloid in the spleen or kidneys and on several others without amyloid. Lipid was found among these amyloid deposits in every case. These deposits were more marked in those rabbits on a high cholesterol diet but were present in those on a diet without egg yolk supplements.

The rabbit (W-231) which served as the impetus for this experiment was fed a high cholesterol diet and received no injections of caseinate. It was sacrificed at the end of 12½ months. Marked atherosclerosis was evident. In addition, massive deposits of amyloid were present in the spleen and kidneys. They stained metachromatically with crystal violet. Massive deposits of lipid were also present among the amyloid aggregates in both organs, both extracellularly and in foam cells.

TABLE I. Autospy Findings - First Series

Animal Number	Length of Experiment	Mode of Exitus	Gross & Histological Findings: Heart	Aorta	Lungs	Spleen	Liver	Kidney	Nodule
C-E 1	259 days	S.	O.P.	M.P.	C. & E.	4+,Fm.C., G.C. Large	F.C.,C.N.	G.A.,C., G.C.	M.,FCP.
C-E 2	17	A.	O.P.	F.P.	C. & E.	0	F.C.	0	0
C-E 3	138	S.	0	M.P.	C. & E.	0	F.S.	F.S.	M.,FCP.
C-E 4	95	S.	O.P.,H.	M.P.	C. & E.	0	Perich.	0	F.,FCP.
C-E 5	52	D.	F.F.	F.P.	C. & E.	PMN	PMN	F.S.	0
C-E 6	69	A.,W.	0	M.P.	0	0	P.C.	0	F.
C-E 7	137	S.	O.P.	M.P.	C. & E.	RCH	0	0	M.,FCP.
C-E 8	246	S.,W.	0	M.P.C.	C. & E.	2+, RCH	F.C.	0	M.,FCP.
C-E 9	54	D.	O.P., F.F.	M.P.C.	C. & E. Abs.	PMN, Abs.	Abs.	Abs.	F.
C-E 10	252	S.	O.P.	M.P.	C. & E.	2+, RCH	F.C., Chol.	0	M.,FCP.
C-E 11	94	D.	F.I.	M.P.	C. & E.	0	Perich.	0	F.,FCP.
C-E 12	131	S.	0	M.P.	0	0	F.C.	0	M.,FCP.
C-C 1	138	S.	0	F.P.	C. & E.	0	0	0	M.,FCP!
C-C 2	104	D.	0	0	C. & E.	PMN	Perich., C.N.,Abs.	0	F.
C-C 3	183	D.	F.F.,H.	0	C. & E.	PMN	Perich., C.N.	GMN,PMN	M.
C-C 4	260	S.	0	0	C. & E.	4+,Fm.C., RCH, G.C.	F.C.	G.A.,C.	M.
C-C 5	37	D.	H.,A.,M.	M.P.C.	C. & E.	0	PMN	Abs.	0
C-C 6	129	D.	F.F.	0	C. & E.	PMN	P.C.	0	M.

Legend: C-E = casein plus egg yolk; C-C = casein alone.

General: Fm.C. - foam cells; G.C. - giant cells; 0 - within normal limits; PMN - post mortem necrosis.

Mode of exitus: A. - died in anaphylactic shock; D. - found dead; S. - sacrificed; W. - wasted prior to death.

Heart: O.P. - partially occluding plaque in coronary artery; H. - arteritis and/or scarring suggestive of hypersensitivity reaction; F.I. - foam cell infiltration of intima; F.F. - focal fibrosis; M. - myocarditis.

Aorta: F.S. - fatty streaks with foam cells; F.P. - few raised plaques; M.P. - many plaques; M.P.C. - many plaques with calcification.

Lungs: C. & E. - congested and edematous; P. - pneumonia.

Spleen: 1-4+ - scale of severity of perifollicular deposition of amorphous eosinophilic deposits (Amyloid); RCH - perifollicular reticulum cell hyperplasia.

Liver: F.C. - fatty change; C.N. - centrolobular necrosis; Perich. - pericholangitis; P.C. - portal cirrhosis; Chol. - cholesteatoma.

Kidney: G.A. - amorphous aggregations (amyloid) in glomeruli; B.M. - basement membrane thickening; C. - casts; F.S. - focal scarring; GMN - acute diffuse glomerulonephritis.

Nodule: F. - few; M. - multiple; FCP - foam cells at periphery of abscess; B. - bacterial colonies lining capsular wall of abscess.

TABLE 2. Autopsy Findings - Second Series

Animal Number	Length of Experiment	Mode of Exitus	Gross & Histological Findings						
			Heart	Aorta	Lungs	Spleen	Liver	Kidney	Nodule
W-G 1	105 days	D.	O.P., F.F.	F.S.	C. & E.,P.	PMN	F.C., Abs.	PMN	M.,B.
W-G 2	23	D.	PMN	0	C. & E.	PMN	PMN	PMN	0
W-G 3	75	D.	H.	0	C. & E.	0	Perich.	PMN	F.
W-G 4	112	D.	0	F.P.	C. & E.	3/,G.C.	Perich., F.C.	0	M.,FCP.
W-G 5	75	D.	0	F.S.	C. & E.	2/	Perich.	B.M.	F.,FCP.
W-G 6	154	S.	O.P.	F.P.	C. & E., H.,P.	RCH	Perich., F.C.,Abs.	0	M.,FCP.
W-G 7	45	A.	H.	0	C. & E.	0	Perich., F.C.	GMN	F.,FCP.
W-G 8	128	D.	0	0	C. & E.	4/	Perich.	PMN	M.,FCP.
W-G 9	154	S.	H.	0	C. & E.	0	Perich.	0	M.
W-G 10	154	S.	H.	0	C. & E.	0	Perich.	G.A.	M.,FCP.
W-G 11	154	S.	H. or O.P.?	0	0	1-2/	Perich.	G.A., B.M.,C.	M.,FCP.
W-G 12	154	S.	0	0	C. & E., Abs.	0	Perich.	0	M.

Legend: same as that used in Table 1.
W-G 1 - 6: casein plus egg yolk; W-G 7 - 12: casein alone.

TABLE 3. Serum Lipid and Protein Determinations

Animal Number	Day of Experiment	Total Cholesterol in mg%	Free Cholesterol in mg%	Fatty Acids in mEq/L	Lipid Phosphorus in mg%	NPN in mg%	Total Protein in Gm%	Albumin in Gm%	Globulin in Gm%
C-E 1	11	290.5	145.2	36.3	17.3	30.8	6.15	4.03	2.12
	259	1,150.0	398.4	76.6	25.0	36.4	5.5	2.40	3.10
C-E 2	No Blood Drawn								
C-E 3	31	705.5	189.3	30.0	13.2	34.6	6.95	3.35	3.60
	138	659.8	226.8	36.6	16.5	39.2	7.21	3.52	3.69
C-E 4	38	357.5	111.2	24.0	11.6	39.2	6.68	4.03	2.65
C-E 5	44	687.5	209.9	42.0	18.2	28.0	7.06	4.60	2.46
C-E 6	50	145.2	71.4	23.3	11.5	26.6	5.61	3.63	1.98
C-E 7	57	516.0	183.4	31.4	15.6	37.0	5.85	2.24	3.61
	137	680.0	306.6	49.1	23.2	39.2	7.21	4.38	2.83
C-E 8	57	524.4	190.9	37.3	16.0	28.0	6.40	4.25	2.15
	240	580.0	139.4	34.0	11.5	50.0	6.93	4.10	2.83
C-E 9	No Blood Drawn								
C-E 10	59	725.0	204.2	28.0	14.7	33.6	6.16	3.81	2.35
	233	700.0	166.0	36.3	13.2	50.0	6.70	3.60	3.10
	252	575.0	182.0	30.2	--	35.8	7.15	3.35	3.80
C-E 11	59	412.5	106.2	22.0	10.6	47.0	6.02	3.40	2.82
C-E 12	59	740.0	223.2	30.0	14.0	25.6	7.30	3.97	3.33
	131	423.3	189.0	30.0	14.0	40.2	6.99	4.38	2.61

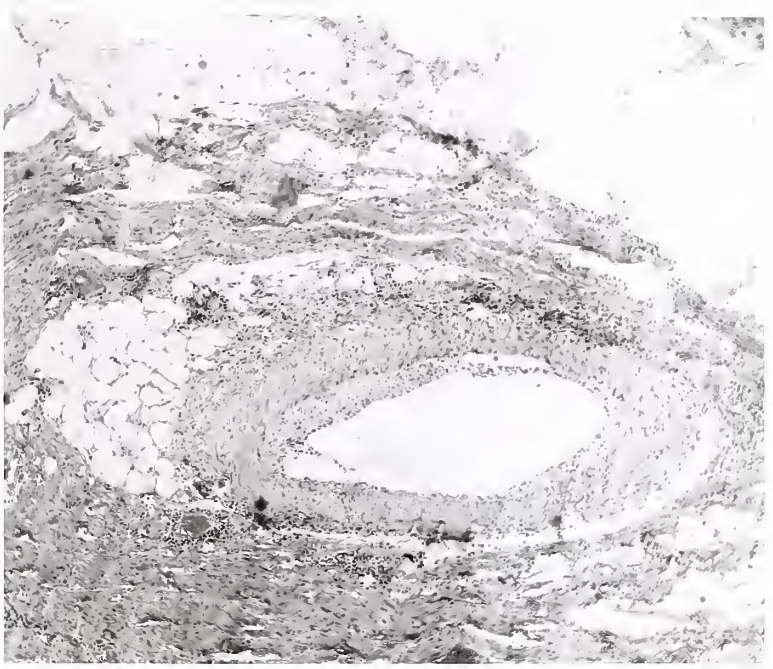
C-C 1	31	122.5	39.8	11.3	3.0	25.6	7.80	3.80	4.00
	138	45.6	12.6	10.0	5.5	33.9	7.34	4.48	2.86
C-C 2	38	44.2	16.0	8.0	3.6	33.6	6.75	4.25	2.50
C-C 3	44	45.6	20.1	10.6	3.5	16.8	7.18	4.80	2.38
C-C 4	50	64.7	25.6	11.3	4.1	28.0	6.30	3.93	2.37
	260	250.0	98.6	23.3	6.5	30.8	6.50	2.69	3.81
C-C 5	No Blood Drawn								
C-C 6	57	97.6	35.5	10.6	5.6	48.0	6.18	4.06	2.12

TABLE 4. Serum Lipid and Protein Determinations

Animal Number	Day of Experiment	Total Cholesterol in mg%	Free Cholesterol in mg%	Fatty Acids in mEq/L	Lipid Phosphorus in mg%	NPN in mg%	Total Protein in Gm%	Albumin in Gm%	Globulin in Gm%
W-G 1	0	51.2	16.0	--	--	33.6	6.04	4.34	1.70
	78	470.0	136.9	27.0	9.2	36.0	6.75	3.90	2.85
W-G 2	0	91.5	25.2	--	--	33.6	5.20	4.37	0.83
W-G 3	0	61.0	25.0	--	--	41.4	5.16	4.23	0.93
W-G 4	0	45.0	17.6	--	--	48.0	6.03	4.23	1.80
	78	475.0	149.0	38.0	14.7	28.0	6.26	4.00	2.26
W-G 5	0	74.0	26.4	--	--	35.8	5.20	4.23	0.97
W-G 6	0	66.0	12.6	--	--	31.2	6.04	4.37	1.67
	78	360.0	112.0	20.0	9.8	26.8	7.30	3.60	3.70
	155	637.5	129.9	73.0	18.0	44.8	7.98	3.69	3.29
W-G 7	0	66.0	30.2	--	--	33.6	5.70	4.20	1.50
W-G 8	0	70.0	32.6	--	--	37.8	6.24	4.23	2.01
	78	205.0	56.4	15.0	5.0	16.8	7.40	3.40	4.00
W-G 9	0	70.0	30.2	--	--	28.0	6.39	4.30	2.09
	78	55.4	16.6	12.0	2.1	18.0	7.50	4.40	3.10
	155	137.9	44.0	22.6	5.0	42.0	7.55	3.80	3.75
W-G 10	0	85.0	37.8	--	--	33.6	5.90	5.50	0.40
	78	837.0	83.0	14.0	5.6	31.4	7.30	3.40	3.90
	155	224.1	56.7	22.6	6.0	39.2	7.72	3.80	3.92
W-G 11	0	92.5	42.0	--	--	39.2	5.50	5.50	0?
	78	637.0	74.0	20.0	6.4	28.0	5.80	3.20	2.60
	155	262.5	98.0	40.0	9.5	42.0	6.10	2.44	3.66
W-G 12	0	95.0	30.1	--	--	41.4	5.50	5.50	0?
	78	662.0	64.7	15.0	5.7	44.8	6.20	3.60	2.60
	155	255.0	64.7	21.3	8.0	39.2	7.00	4.20	2.80

Normal values: 23

Mean -	45	22	6.1	6.5	18	5.7	4.2	1.5
Range -	10-80	0-47	--	4.8-8.7	--	--	--	--



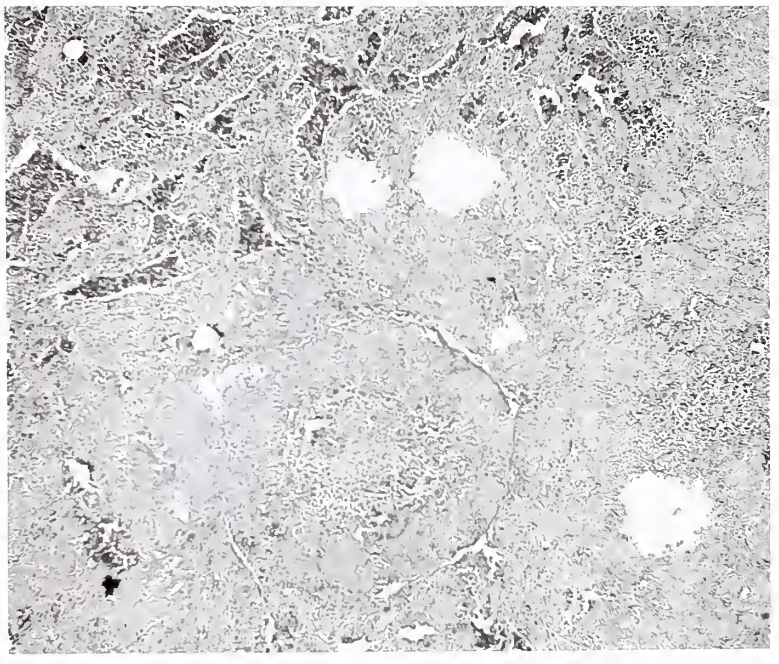


Fig. 1. Rabbit W-G 7. Hematoxylin and eosin stain. Coronary artery with extensive periarteritis and endarteritis. x53.

Fig. 2. Rabbit C-E 1. Hematoxylin and eosin stain. Massive deposits of amyloid replacing normal tissue in spleen. x125.

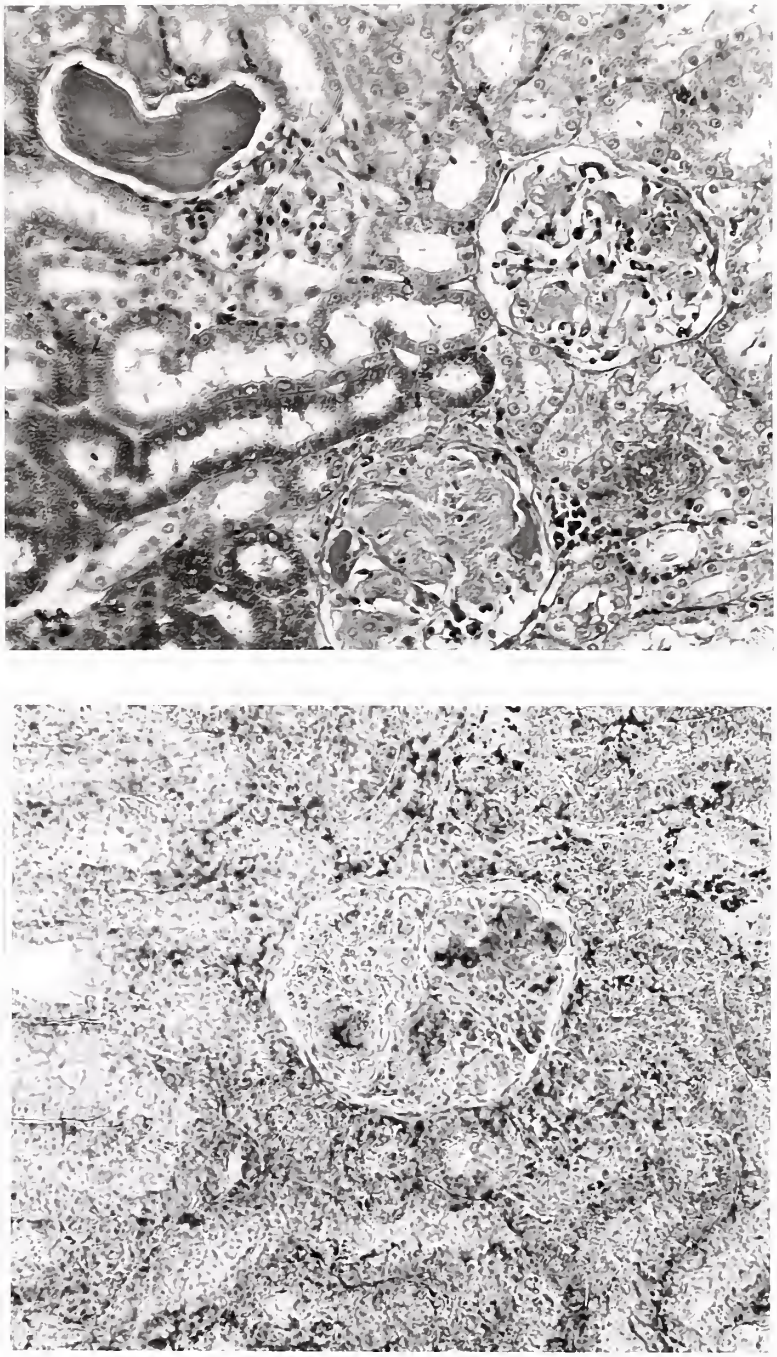


Fig. 3. Rabbit W-G 11. Hematoxylin and eosin stain. Kidney with focal deposits of amyloid in glomeruli and with tubular casts. x260.

Fig. 4. Rabbit W-G 11. Sudan IV stain. Focal deposition of lipid in glomeruli in areas where amyloid is seen in Fig. 3. x260.

DISCUSSION

Amyloidosis occurred in 9 of 30 rabbits. The fact that the amyloid deposits did not occur in as many rabbits as predicted according to Cohen et al [17] and stained variably leads us to inquire into the exact nature of amyloid, its variable staining characteristics, and the pathogenesis of the disease process in man and in experimental animals.

Amyloid is usually described as a glycoprotein or more specifically, a globulin and an acid mucopolysaccharide which form an insoluble, enzyme resistant complex. [24] The difficulty in determining the exact chemical structure of amyloid is in part due to the problem of extracting amyloid from the tissues. [25] Nevertheless, protein is the predominant organic component. The amino acid composition is non-specific. [26] Electrophoretic identity of the protein component is difficult to determine because of the denaturation that occurs in the process of analysis. [25] Tyrosine is present and thereby, distinguishes it from hyalinized connective tissue in which it is absent. The tryptophan is about the same in amyloid and hyaline. Amyloid is 82.8 percent water by one analysis. [25]

The carbohydrate present in amyloid is slightly over 4 percent. [24] About half of this is a hexosamine, mainly glucosamine, and half is neutral sugar, primarily galactose, but also glucose, mannose, and fucose. Smaller amounts of sialic acid are present. Uronic acid and sulfate are present in concentrations equal to those of the surrounding tissue. Hydroxyproline makes up less than 1 percent of the dry weight. [26] It should be noted that this pattern of carbohydrates present in amyloid is similar to that in many tissues and serum. Therefore, these details do not aid in establishing the distinct biochemical nature of amyloid. [25]

It is relevant that lipids are not usually present in excessive amounts in amyloid deposits. Nevertheless, many extracellularly deposited proteins contain variable, yet small amounts of lipid. [24]

The serum determinations in Tables 3 and 4 in this experiment reveal a consistent elevation of globulin levels with reversal of the A/G ratio in the majority of rabbits with amyloid. The question that logically arises from this finding of elevated serum globulin levels concerns its relationship to the material at the sites of deposition, and hence, etiology. The levels of globulin did not always correlate with the extent of involvement. In this connection it is interesting to note the rarity of amyloidosis in individuals with kala-azar or lyphmogranuloma venereum inspite of very high plasma globulin levels. [7] Electrophoretic patterns were not done in the present experiments. However, in man these usually show an elevation of alpha - 1 and alpha - 2 globulins, the latter being more prominent when nephrosis complicates the picture. [25] In primary amyloidosis, Rukavina et al. [27] found an abnormal globulin peak between the alpha - 2 and beta globulins. However, this report was inconclusive since the abnormal globulin was found in unaffected family members as well as those with amyloidosis. Thus, the existence of an unassociated genetic defect as manifested by the abnormal globulin could not be ruled out. Nevertheless, a later report [28] confirmed the existence of an elevated level of alpha - 2 globulins in amyloidosis.

Rukavina et al. also found increased quantities of serum beta lipoproteins, Most of their cases (82 percent) had elevated lipoproteins in the -S 70, -S 40-70, -S 25-40, -S 20-25, -S 1-10 classes. However, only a few (15 percent) had elevated serum cholesterol levels. In our studies, the total cholesterol, free cholesterol, fatty acids and lipid phosphorus levels were all elevated in the cholesterol-fed rabbits and in rabbits C-C-4 and W-G-8, 10, and 11 which had evidence of amyloid infiltration. The elevated levels in the cholesterol fed animals was expected, but those in the other rabbits were not. Thus, a derangement of lipid metabolism in experimental amyloidosis is suggested. Serum non-protein nitrogen values were

slightly elevated throughout. These values, however, were not markedly elevated and did not correlate with the incidence of amyloidosis or with the severity of the lesions.

When amyloid is present in humans in massive quantities, it is smooth and waxy rendering the organ involved a rubbery texture. [6] Grossly, it can be identified by the blue to black color the tissue containing it turns to when treated with iodine and dilute sulfuric acid, as originally described by Virchow. [29] Hematoxylin and eosin staining will reveal an amorphous, hyaline, eosinophilic material microscopically. Yet, small deposits and other eosinophilic deposits may be difficult to distinguish unless special staining techniques are employed. Furthermore, the deposits seen in primary amyloidosis are even more difficult to differentiate with special stains than are those in the secondary variety.

Of the more specific stains for amyloid, Congo red and crystal violet are the most commonly used. Congo red has an unexplained affinity for amyloid and stains it brick red. However, other hyaline compounds may stain a similar color. A more specific stain for amyloid is methyl violet or crystal violet. The material if positive will stain deep pink to red while the surrounding tissues stain blue. This metachromasia, that is, the ability of a dye to produce a color on staining tissue which is different from the original dye is distinctive for amyloid and other acidic compounds of high molecular weight. [30] The main difficulty with this stain is that fading occurs with paraffin sections and they stain less well than fresh, frozen sections. Nevertheless, the methyl violet and crystal violet stains remain the most specific stains for amyloid.

Other stains may be used to characterize the various chemical components of amyloid. The periodic acid-Schiff (PAS) reaction affects the 1:2 glycol groups in tissues, that is, carbohydrates. When a specimen stains magenta or purple it is

PAS-positive. Mucoproteins and neutral mucopolysaccharides stain deep purplish red while glycoproteins are a paler red or pink. In practice, this distinction is not always evident. The van Gieson stain will cause amyloid to become gray-yellow thus distinguishing it from collagen which is red. Yet, fibrin, smooth muscle and neuroglial fibrils take the same stain as amyloid. Phosphotungstic acid and hematoxylin (PTH) will stain amyloid reddish-orange as opposed to the blue color of fibrin. With the Masson trichrome stain, amyloid is said to stain gray indicating its globulin content; collagen and mucin stain green while fibrin is red. Of all these stains, the ones in which the amyloid stains a distinctly different color from the surrounding tissue is the most suitable; hence, the popularity of the metachromatic stains. Nevertheless, these stains have their disadvantages as noted and are delicate to use in practice. The stains that rely on variations in the shade of one color, Congo red for example, are not useful to the unsophisticated eye in other than obvious cases. A combination of hematoxylin and eosin, crystal violet stains and one other - Congo red or van Gieson - should be sufficient for detecting amyloid. In the present experiments, the hematoxylin and eosin stain was helpful the most. The crystal violet stain was useful when viewed immediately after the stain was made and not fifteen minutes later. The Congo red stain was not as helpful as is usually claimed.

Awareness of the chemical nature and the staining characteristics of amyloid might furnish leads for the pathogenesis of amyloidosis. Questions which might be asked are as follows: How do these deposits form? Do they originate in the organ in which they are seen or do they get to these organs from the blood stream by filtration through the walls of arteries, arterioles or capillaries? Is this deposit a reaction of the body to a specific disease process such as, an antigen-antibody reaction?

The first major theory as to the pathogenesis of amyloidosis concerns the

suggestion that a primary local defect of mesenchymal tissue or fibroblasts leads to the deposition of this abnormal glycoprotein. Although the defect occurs at the site of deposition the defect is thought to be a generalized disorder of fibroblastic elements according to Reiman [31] or a generalized dysfunction of mesenchymal tissues as proposed by Warren. [32] When one examines the histological sections, one is impressed by the abundance of hyperplastic reticulum cells in some instances. Do these cells indicate that amyloid is being secreted as suggested by Warren and by Reiman or is the amyloid being phagocytized by these cells after being deposited by the blood stream?

Teilum in a series of articles [33,34,35] attempts to show that reticuloendothelial cells contain a large amount of globular or finely granular PAS-positive material in the cytoplasm. He considers this material to be a mucoprotein or glycoprotein synthesized by the cell in response to various, non-specific, antigenic stimuli. He even goes so far as to distinguish functional stages in the process of amyloid production as determined by the cellular content of PAS-positive material (glycoprotein) as opposed to pyroninophilic material (ribonucleic acid) in the cytoplasm. He shows that as the PAS-positive material increases the pyronine-positive reaction of the cytoplasm decreases. This observation has led him to conclude that synthesis and not absorption is the work of these proliferating reticulum cells.

The other two major theories consider that the amyloid reaches the parenchymal tissues via the blood stream. Evidence for this is presented by Larson. [35] He demonstrated that deposits of amyloid are continuous with similar deposits around the endothelium of the venocapillary. He proposed that alterations in the permeability of venous endothelium was responsible for amyloid deposition from intercellular lymph. In our own experiment, amyloid deposits were occasionally found

subendothelially on the arterial side of the circulation. This is a known and characteristic site for amyloid to be deposited and raises the possibility of filtration of the material from the blood stream through the arterial wall. A similar process is often invoked for the path of beta-lipoproteins in atherosclerosis, as will be discussed later.

The difference between these last two hypotheses is in the concept of the nature of the precipitate. One states that the deposit is due to hypersensitivity and is the result of an antigen-antibody reaction. The other considers amyloid to be a non-antibody, abnormal protein complex. That this compound is partially antibody was suggested though not established by Vasquez using the fluorescent antibody techniques of Coons. [37] But again, whether the deposit is a complex of circulating antibody with tissue antigen or a complex of tissue antigen and antibody produced by reticulo-endothelial cells in this tissue is still not answered.

Evidence for an antigen-antibody reaction as the etiological factor is further suggested by the presence of the lesions of hypersensitivity in our experimental animals. Arteritis of the hypersensitivity type was seen in 9 rabbits none of which survived longer than 2½ months. The lesions were originally shown to be associated with a hypersensitivity state by Rich. [38] He demonstrated that the lesions of periarteritis nodosa in rabbits follow large doses of horse serum and were not the result of infection. A wide variety of other foreign proteins, such as penicillin, sulfonamides, DDT, Dilantin, and iodides can produce these lesions. That casein may give rise to lesions of hypersensitivity is a distinct possibility and should be considered strongly in our thinking about this disease process.

Long-term stimulation of these hyperimmune mechanisms may give rise to amyloid deposits. However, in our series, the etiological role of infection should be

discussed. The problem has been considered previously. Letterer [39,40] used injections of ribonucleic acid to induce amyloidosis, but felt the disease was secondary to infection from unsterile injections as evidenced by abscess formation at injection sites. In nearly every rabbit in our series, nodules formed on the backs at sites of injection. Microscopically, these proved to be abscesses with a fibrous capsule surrounding inflammatory cells and necrotic debris. Only rarely were clumps of bacteria seen in these abscesses. Percutaneous biopsy of these nodules with rigid precautions for sterility failed to grow out organisms except in a few instances as noted. Some animals (rabbits C-E-9 and C-C-2) died with evidence of a septicemia or other infectious process. Yet, the animals with amyloid in their spleens did not have any signs of an infectious process. The nodules on their backs were probably sterile abscesses, except in a few instances. Therefore, the role of infection as a pathogenic agent for amyloidosis in our series is unlikely.

The fact that amyloid can appear either secondary to some chronic destructive process or arise "de novo" raises the question of the relation between primary and secondary amyloidosis. The primary type in man is usually described as a disorder of mesenchymatous tissues affecting mainly the blood vessels, heart, tongue, gastrointestinal tract and kidneys. These tissues do not usually stain with Congo red or metachromatically. The secondary variety is designated as a disease of parenchymatous organs involving the spleen, liver, kidneys, and adrenal glands. [6] These deposits will stain characteristically with crystal violet and Congo red. This often made, clear-cut distinction is most misleading. When all tissues are studied for amyloid, involvement of other organs in addition to those expected for the type are found. Thus, in a person with so-called primary amyloidosis, the spleen, liver, and kidneys may be infiltrated with amyloid and may exhibit metachromasia and take up Congo red. Likewise, those with secondary amyloidosis may have involvement of numerous blood vessels and the tissues may

not exhibit the usual staining characteristics.

The physical structures of the deposits from the primary and secondary type are similar. In the polarizing microscope, positive birefringence in the direction of the long axis of the amyloid deposit is seen. [17] This suggests that the material is an organized structure rather than an amorphous mass. Under the electron microscope, amyloid is seen to contain fibrils that differ from those seen in collagen. [41,42]

These deposits in primary and secondary amyloidosis are similar not only morphologically, histochemically, and structurally, but also biochemically as might have been predicted with the foregoing information. On chemical analysis of the protein and carbohydrate moieties, similar results are obtained. [8,25] It should be kept in mind that although these compounds are similar they are not identical. That is, not even amyloid from two patients or two animals with secondary amyloidosis are identical on chemical analysis, though the component parts are approximately alike. In the serum of patients with the primary or secondary variety, alpha - 2 globulins and hexosamines are often elevated; thus, demonstrating another similarity.

In review of the biochemical and histochemical findings and the theories on pathogenesis, we might consider the following guide lines to help us view further research in this field. Biochemically, amyloid is a macromolecular compound, a glycoprotein that is about 4-5 percent carbohydrate, 15-20 percent protein and the remaining, water. Carbohydrate analysis, thus far, reveals nothing that would differentiate amyloid from other glycoproteins of the body. Protein fractionation is at best difficult. Yet, the deposits are usually associated with elevated serum alpha - 2 globulins, hexosamines, and lipoproteins. Structurally, amyloid presents not as an amorphous mass but as a fibrillar structure of a unique character. Histochemically, this hyaline eosinophilic material may or may not

stain metachromatically with crystal violet or stain with Congo red. Other special stains may be helpful, such as, PAS and van Gieson. The pathogenesis of this disorder is still poorly understood. What is clear, however, is that primary and secondary amyloidosis are not distinct entities but more likely variations in one continuous spectrum depending on the inciting process. In view of this evidence, we might reasonably consider the process of deposition to be one in which a circulating macromolecule is formed following a chronic stimulus whether it be associated with infections, certain tumors, or injections of foreign protein. The body reacts to one of these stimuli in a hyperimmune manner with the deposition of an antigen-antibody complex at the site seen histologically. The reticulo-endothelial system attempts to phagocytize these deposits but is eventually over-whelmed. Following this point, the normal tissue is gradually replaced by amyloid.

In order to compare the pathological processes of amyloidosis and atherosclerosis, we will consider briefly the pertinent biochemistry, histology, and pathogenic mechanisms of atherosclerosis. No extensive review will be attempted.

The substance that usually plays a major role in atherosclerosis, is the family of serum beta-lipoproteins. Several fractions of this group have been implicated in coronary artery disease. Gofman suggested there are specific atherogenic lipoproteins, namely, S_f 12-20. [43] Patients with idiopathic hyperlipemia have elevated beta-lipoproteins, primarily in the S_f 20-400 range. Those with idiopathic hypercholesterolemia have increased levels of S_f 0-12 lipoproteins. In the latter patients, the triglycerides are the major component of the lipid present while in the former patients, the cholesterol and cholesterol esters comprise the larger part of the lipid in the serum. Nevertheless, the general feeling at present is that the whole range of beta-lipoprotein fractions is atherogenic. Furthermore, most of these fractions have been found in human atheromas. [44]

The morphology of the various stages of atherosclerosis is usually recognized

with ease under the microscope when stained with routine hematoxylin and eosin. The specific location of the lipid within the cells and among the fibrous tissue in these lesions is identified by the Sudan III and IV or oil red O stains. [30] These stains, however, do not distinguish among the different classes of lipids.

Pathogenetically, we are concerned with the process of imbibition and its relation to local factors of injury. The imbibition hypothesis was originally proposed by Virchow, and later elaborated on by Aschoff. [45] It states that lipids filter through the vessel wall from the plasma and accumulate in the intima and subintimal tissues when various local factors in the wall itself are altered from their normal state. Several methods have been employed to establish the imbibition theory on firm ground, namely: the demonstration of macromolecular filtration through the endothelium and the entire wall of the aorta; [44] the intravenous injection of radioisotope labelled cholesterol with subsequent localization in the atheroma; [46,47] the finding of the chemical similarity of the constellation of lipids seen in the serum and in the atheroma; and finally, the existence of these low density, beta-lipoproteins in the diseased aorta as opposed to their absence in the normal part of the aorta.[48]The significance of local factors in altering the permeability of the vessel wall to these beta-lipoproteins has been emphasized by Duff [49] and Waters. [50] In these articles, anoxia, infection and other chemical toxins have been shown to induce focal changes in the arterial wall which in turn probably alter the vessel permeability to large plasma molecules such as lipids and also, favor their precipitation.

Two other hypotheses on the pathogenesis of atherosclerosis should be mentioned. One attempts to explain the origin of the foam cell. This theory has been propounded by Leary who felt he had demonstrated that foam cells circulate in the blood stream and then invade the subendothelial layer of the artery by penetrating the intima. [51] The other theory is the incrustation theory suggested

by Rokitansky in 1852, and later revived by Duguid. [52] It states that thrombotic material deposits on the intima and initiates the atherosclerotic process.

With the theories for the pathogenic mechanisms of amyloidosis and atherosclerosis in mind, we shall now explore the relationship between these two diseases.

Filtration of molecules through the endothelium of blood vessels followed by deposition in the arterial wall or in the parenchymal tissues figures prominently in the theories on the etiology of both diseas. In atherosclerosis, beta-lipoproteins are thought to deposit in the intima of large arteries after they filter through the endothelium. In amyloidosis, the agent that deposits has not been identified and analysed as well. But, one of the more prominent theories considers that an abnormal protein, whether it be an antigen-antibody complex, an antigen or an antibody alone may filter through the endothelium and deposit in the parenchymal tissues. Thus, the mode of transportation of the abnormal deposits in either disease may be similar.

In this experiment as in other research on atherosclerosis, lipid has been amply demonstrated with the Sudan IV stain in the intima of large arteries, as well as in numerous organs, such as the spleen, liver, and kidney. [53] However, lipid was also demonstrated in the spleen and kidneys of rabbits on low cholesterol diets. The lipid in both instances was located extracellularly and within lipophages. Whether the amyloid accompanies or precedes the lipid deposits in the parenchyma is not clear yet. Nevertheless, a disorder of lipid metabolism in amyloidosis is demonstrated by the elevated serum lipid levels as well as by these deposits in the spleen and kidneys of amyloid-involved rabbits. These findings were unexpected in rabbits on a diet low in cholesterol.

There was little correlation between the lipid levels in the serum and the

severity of amyloid involvement. It was unusual to find deposits of amyloid in the arteriolar wall as opposed to the frequent deposits classically described. The absence of these deposits in the wall of the artery mitigates somewhat the argument for filtration of amyloid or a related material through the vessel wall.

The finding of elevated serum lipid levels and of lipid deposition along with the amyloid in the same sites strongly suggests that a disorder of lipid metabolism is associated with amyloidosis. That amyloid deposits reach the parenchyma in a similar way that beta-lipoproteins deposit in the intima of large arteries and in parenchymal organs is a postulate that may be deduced from the literature discussed. In this experiment the intermingling of lipid and amyloid in the spleen and in the renal glomeruli may be used as evidence to show that atherosclerosis and amyloidosis may have parallel pathogenetic mechanisms.

SUMMARY AND CONCLUSIONS

1) Amyloidosis occurred in 9 out of 30 rabbits when injected subcutaneously with a solution of sodium caseinate.

2) The disease occurred more frequently and at an earlier time when the concentration of the solution was increased from 10 percent to 15 percent. The incidence of experimental amyloidosis reported by Cohen *et al* was not achieved.

3) A number of casein-injected rabbits either on a high or a low cholesterol diet developed elevated serum lipid levels and massive deposits of lipid in areas of amyloid accumulation. An associated disorder of lipid metabolism is thereby suggested to occur in amyloidosis.

4) Deposits of amyloid stained with difficulty using crystal violet and Congo red, even though they were characteristic with the hematoxylin and eosin stain. The crystal violet stain was reliable only when viewed within fifteen minutes after the stain was done. Congo red was not a reliable stain.

5) Amyloid deposits occurred in the spleen and kidneys only and were associated with elevated serum globulins and often with a reversal of the albumin/globulin ratio.

6) Approximately one-half of the rabbits died during the experiment. A number of these exhibited vascular lesions of a hypersensitivity type. The role of infection was probably not of major importance. Abscesses developed at injection sites but were usually sterile. The role of hypersensitivity to casein or its congeners should now be considered in protein-induced amyloidosis. Previous reports of hypersensitivity lesions in this experiment model have not been found in the literature.

7) A brief review of the literature on the pathogenesis, biochemistry, and histology of amyloidosis and related aspects of atherosclerosis was made. The relation of the pathogenesis of experimental amyloidosis to that in experimental atherosclerosis is discussed, especially as concerns the filtration of beta-lipoproteins and amyloid or related substances through the vessel or capillary wall with subsequent deposition in the vascular wall or organ parenchyma.

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